



Pyrosequencing analysis for characterization of soil bacterial populations as affected by an integrated livestock-cotton production system

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ARTICLE INFO

Article history:

Received 17 July 2009

Received in revised form 15 January 2010

Accepted 19 January 2010

Keywords:

16S rRNA

Pyrosequencing

Grazing

Soil management

Enzyme activities

Microbial community

ABSTRACT

Bacterial tag-encoded FLX amplicon pyrosequencing of the 16S rDNA gene was used to evaluate bacterial diversity of a clay loam soil (fine, mixed, thermic Torrertic Paleustolls) after 10 years under an integrated livestock (beef)-cotton (*Gossypium hirsutum* L.) production system compared to continuous cotton in a semiarid region. In the integrated system, cattle alternatively grazed a perennial warm-season grass [*Bothriochloa bladhii* (Retz) S.T. Blake] paddock and small grains grown in two paddocks of a wheat (*Triticum aestivum* L.)-fallow-rye (*Secale cereal* L.)-cotton rotation. Areas excluded from grazing in the integrated system were also evaluated. Maximum observed number of unique sequences operational taxonomic units (OTU) at 3% dissimilarity level (roughly corresponding to the species level) corresponded to 1200 and 1100 at 0–5 and 5–15 cm depths, respectively. Predominant phyla (up to 65% of abundance) at 0–5 and 5–15 cm in this soil were *Proteobacteria*, *Actinobacteria* and *Gemmatimonadetes*. *Proteobacteria* were predominant in soil under all components of the integrated livestock-cotton system compared to continuous cotton whereas *Bacteroidetes* were predominant under continuous cotton. *Firmicutes* (i.e., *Clostridia*) and *Chloroflexi* (i.e., *Thermomicrobia*) were more abundant in soil under fallow periods of the rotation compared to under cotton (Rye-Cotton-Wheat-Fallow or continuous cotton) or grass (i.e., pasture). The lowest OTUs were detected in soil under fallow periods of the rotation (Wheat-Fallow-Rye-Cotton) compared to the other treatments. Grazing effects were significant for *Actinobacteria*, *Proteobacteria* and *Chloroflexi*. Compared to the continuous cotton system, this study revealed significant changes in bacterial phyla distribution under integrated livestock-cotton systems for a semiarid soil after 10 years. Positive correlations were found between certain bacteria (*Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Verrucomicrobiae* and *Fibrobacteres*) and the activities of alkaline phosphatase and β -glucosidase or β -glucosaminidase.

Published by Elsevier B.V.

1. Introduction

Efforts to sustain cotton production in the Texas High Plains semiarid region have focused on integrated livestock-crop production systems in order to reduce irrigation needs relative to typical practice of continuous cotton monoculture (TAWC, 2007; Allen et al., 2005, 2008). Integrated livestock-crop production systems compared to monoculture crop production have shown to reduce water usage, fuel costs associated with irrigation, and potentially improve several soil quality parameters (Franzluebbers, 2007). Therefore, a unique long-term research was initiated in the Texas High Plains in 1997 to compare an integrated livestock (beef)-cotton production system and the typical practice of

continuous cotton monoculture. In the integrated system, cattle alternatively grazed a perennial warm-season grass paddock and small grains grown in two paddocks with two stages of the same rotation. In addition, non-grazed zones were also established within each paddock area to evaluate long-term soil responses to the effects of grazing in the integrated system. Our research is focused on evaluating the microbial community size, structure and activity of soil as indicators of soil quality and functional changes as affected by this integrated livestock-cotton system in the Texas High Plains research site.

The first soil quality study conducted during year 5 showed that soil under the perennial grass was higher in organic C, aggregate stability, microbial biomass C, enzyme activities and fatty acid methyl ester (FAME) indicators for protozoan and fungal populations than soil in the continuous monoculture cotton at 0–5 cm (Acosta-Martínez et al., 2004). Differences were also found in the cotton-small grain rotation within the integrated system, but

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depended on the stage of the rotation. Thus, the perennial pasture was already differentiated from continuous monoculture cotton, but this was not the case with the rotations after 5 years. In a study after 10 years, the rotation showed higher total C, microbial biomass C and N, and several enzyme activities of C, N and P cycling compared to continuous cotton despite when the samples were taken (Acosta-Martínez et al., submitted for publication). Currently, the specific responses of the soil bacterial populations to the integrated livestock-cotton system are still not well understood. Bacterial populations play critical roles in soil biogeochemical processes as they are more diverse and ubiquitous than any other microbial group in soil. Bacteria act as a buffer in the soil ecosystem due to their key role in soil processes including nutrient cycling, atmospheric nitrogen fixation, and the decomposition of organic materials and/or pollutants. Studies have identified bacteria as very resistant to soil tillage perturbations after a one time tillage event (Wortmann et al., 2008) and to environmental conditions including temperature fluctuations (Dunn et al., 1985; D'Ascoli et al., 2005). Determining any changes at the species level in the soil bacterial populations following implementation of this integrated livestock-cotton production system is crucial for a more complete evaluation of changes in ecosystem health and functioning.

Recent advances in sequencing technology, such as the development of pyrosequencing, for evaluating the bacterial diversity of a variety of samples has been described as promising to be less labor intensive in characterizing microbial community structure in natural environments compared with traditional molecular biological techniques involving cloning (Cristea-Fernstrom et al., 2007; Huse et al., 2007; Liu et al., 2007; Roesch et al., 2007; Sundquist et al., 2007; Acosta-Martínez et al., 2008; Fulthorpe et al., 2008; Cardenas and Tiedje, 2008). Pyrosequencing provides a fast and massive sequencing approach (i.e., 400–600 MB output per run in a day), which can provide between 4500 and 52,000 unique sequences or operational taxonomic units (OTUs; roughly corresponding to the species level) in soils. This output is between 10 and 100 times higher compared to what can be obtained (i.e., about 335 OTUs) using traditional cloning techniques (Roesch et al., 2007; Acosta-Martínez et al., 2008; Jangid et al., 2008). Thus, pyrosequencing is a promising new tool that will expand our understanding of the microbial community structure of soils quickly and more comprehensively than other molecular approaches currently in use. Previously, Roesch et al. (2007) enumerated and contrasted bacterial diversity of four soils from the western hemisphere using a pyrosequencing approach. Later, we used a modification of pyrosequencing to provide information on the bacterial diversity of a single soil as affected by management and land use (Acosta-Martínez et al., 2008). Our approach is a bacterial tag-encoded FLX amplicon pyrosequencing protocol followed by sequencing with the 454 Genome Sequencer FLX System (Roche, Nutley, New Jersey) originally described by Dowd et al. (2008a,b).

The first objective of this study was to apply our pyrosequencing approach (Acosta-Martínez et al., 2008) to determine soil bacterial phyla distribution in the integrated livestock (beef)-cotton production system compared to the continuous cotton monoculture system in the Texas High Plains long-term research after 10 years. In addition, the effects of grazing on soil bacterial phyla distribution were evaluated by comparing the grazed areas and non-grazed areas within the integrated livestock-cotton system. Our second objective was to compare the soil bacterial diversity in the integrated livestock (beef)-cotton production system (grazed and non-grazed areas) and the continuous cotton system using rarefaction curves and different diversity indexes. In addition, in an effort to gain more insight into the bacteria participating in soil metabolic functioning, our third objective was

to explore the relationship of bacterial phyla distribution with selected soil properties including microbial biomass C, total C and N, fatty acids indicators for bacterial groups (G+, G– and actinomycetes) and enzyme activities of C, N or P cycling.

2. Materials and methods

2.1. Experimental design and soil management

The research site was initiated in 1997 at the Texas Tech University field laboratory located in northeast Lubbock County in the Texas High Plains [101°47'W; 33°45'N; 993 m elevation]. The landscape was characterized by nearly level soils with 0–1% slopes. The soil was Pullman clay loam (fine, mixed, thermic Torrertic Paleustolls) with an average pH of 7.4, 38% clay, 28% silt and 34% sand. The research site was defined by a dry steppe climate with mild winters. Mean annual precipitation in this region is 465 mm with most of the precipitation occurring from April through October. The research site compared a continuous cotton system and an integrated cotton and beef cattle system with a paddock for perennial pasture and two paddocks to provide two stages of the same rotation for livestock grazing (Fig. 1). The systems were replicated three times in a randomized block design with a total experimental land area of 12.75 ha. Both systems were irrigated with an underground drip irrigation system with tapes located on 1-m centers and buried about 0.36 m deep. More details are given in Allen et al. (2005).

Each replication of the continuous cotton system comprised a single 0.25-ha plot. Cattle were not present in this system. The cotton varieties have been Paymaster 2326RR (1998–2002), Fibermax 989 BR03-04 (2003–2004), Fibermax 960 BR05 (2005) and Fibermax 9058F (2007). Nitrogen was applied through the irrigation tape during the growing season (mean N application rate was 150 kg ha⁻¹). Phosphorus, potassium, and sulfur were applied by soil test recommendations. No other fertilizers were applied. Chemicals, herbicides, and plant growth regulators were applied according to recommendation of integrated pest management specialists.

Each replicate of the 3-paddock integrated livestock-crop production system included 4 ha (Fig. 1). About 53.6% (2.14 ha) was in the perennial warm-season grass 'WW-B. Dahl' old world bluestem [*Bothriochloa bladhii* (Retz) S.T. Blake] paddock. The remaining 46.4% was equally divided into 2-paddocks for alternate

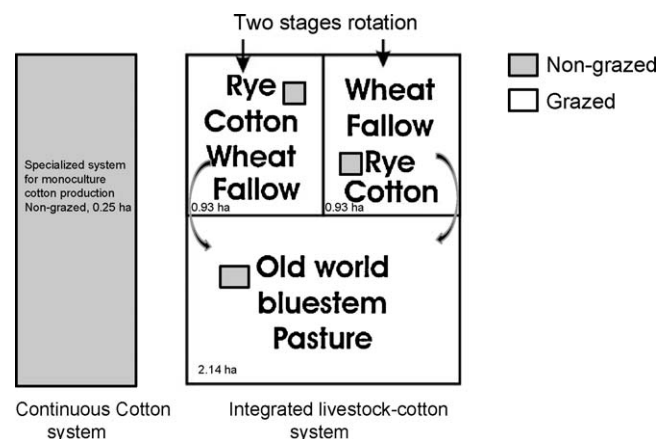


Fig. 1. Experimental design used for the continuous cotton system and integrated cotton and beef cattle system (one field replicate is shown). The integrated system contains a paddock for perennial pasture and two paddocks with two stages of the same rotation for livestock grazing. Cattle alternatively grazed the pasture and rotation within the integrated system (grazed areas), except for excluded caged areas (4.8 m × 4.8 m non-grazed area). Livestock was not a part of the continuous cotton system.

rotation of cotton and small grains (0.93 ha each). Briefly, 'Maton' rye (*Secale cereal* L.), planted in early September, was grazed by Angus and Angus-cross beef steers (*Bos taurus*) in sequence with dormant stockpiled old world bluestem from January until mid- to late-April. Following grazing, rye was terminated with glyphosate and cotton was no-till planted into rye residue. Cotton varieties, fertilization and other chemicals were as described above. After cotton harvest in late autumn, 'Lockett' wheat (*Triticum aestivum* L.) was planted no-till into cotton residue. Wheat was grazed out the following spring prior to again grazing the old world bluestem until mid-July when all steers were removed from the system. Following graze-out of wheat, land was fallowed until rye was planted in September. When the rotation was under fallow, the paddock was sprayed with glyphosate to terminate weeds and was clipped at least once to control volunteer cotton. About 80 kg ha⁻¹ N was applied once each spring to the old world bluestem pastures. Both rye and wheat were fertilized with about 67 kg N ha⁻¹ each year. In summary, perennial pasture provided grazing intermittently from January to mid-July (and a seed crop in October), and then, steers would graze in sequence both rye and wheat as these forages were available for grazing.

At the beginning of the experiment in 1998 (year 1), prior to cattle entering the system, permanently located cages (4.8 m × 4.8 m) were installed in each paddock to prevent animal impact but were managed in all other ways as grazed areas. At the end of each grazing sequence, cages were removed for planting and harvesting and were then reinstalled prior to the next grazing season. While forages within the pasture were grazed, forages within excluded areas were managed as a hay crop.

2.2. Soil sampling

Soil surface samples were taken in July 2007 from the integrated crop-livestock and continuous cotton systems. One composite soil sample was taken by mixing three randomly selected locations (within a 3 m radius) at each of the three field replications of the systems. The samples were sub-divided into 0–5 and 5–15 cm depths. In order to compare the effects of grazing on the soil microbial component within the integrated crop-livestock system, soil samples were taken from both grazed areas and non-grazed caged areas. When crops were present, soil samples were combined from rhizosphere and non-rhizosphere locations.

2.3. Selected soil properties

Soil pH was determined on air-dried subsamples (sieved to <5 mm) using a glass combination electrode with a soil:water ratio of 1:2.5. Soil total C and N contents were determined in subsamples of air-dried soil (sieved to <180 µm) by automated dry combustion (LECO TruSpec CN, Joseph, MI, USA) in a private laboratory (Ward Laboratories, Nebraska, USA). Soil microbial biomass C (MBC) and N (MBN) were determined on a 15 g oven-dry equivalent field-moist soil sample (sieved to <5 mm) by the chloroform-fumigation-extraction method (Brookes et al., 1985; Jenkinson, 1988; Vance et al., 1987) as previously described by Acosta-Martínez et al. (2004). Microbial community structure was evaluated using the fatty acid methyl ester (FAME) following the MIDI (Microbial ID, Inc., Newark, DE, USA) protocol as previously applied to soils (Acosta-Martínez et al., 2004). Relative abundance for different microbial groups was determined using FAMES indicators of fungal populations (16:1ω5c, 18:3ω6c, 18:1ω9c and 18:2ω6c) and bacterial populations including G+ (i15:0, a15:0, i17:0, a17:0), G– (cy17:0, cy19:0, i13:0 3OH and i17:0 3OH) and actinomycetes (10Me 16:0, 10Me17:0 and 10Me 18:0). Enzyme activities important for C (β-glucosidase), C and N (β-glucosaminidase) and P (i.e., alkaline phosphatase) cycling were evaluated

using 1 g of air-dried soil (<5 mm) with their appropriate substrate and incubated for 1 h (37 °C) at their optimal pH as described previously (Tabatabai, 1994 or Parham and Deng, 2000 for β-glucosaminidase activity).

2.4. Soil DNA extraction and sequencing PCR for bacterial tag-encoded FLX amplicon pyrosequencing

DNA was extracted from approximately 0.5 g of soil (oven-dry basis of field-moist soil) using the Fast DNA Spin Kit for soil (QBIogene, Carlsbad, CA, USA) following the manufacturer's instructions. The DNA extracted (1 µl) was quantified using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The integrity of the DNA extracted from the soils was confirmed by running DNA extracts on 0.8% agarose gel with 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). All DNA samples were diluted to 100 ng/µl and 100 ng (1 µl) DNA aliquots of each sample were used for a 50 µl PCR reaction. The 16S universal Eubacterial primers 530F (5'-GTG CCA GCM GCN GCG G) and 1100R (5'-GGG TTN CGN TCG TTG) were used for amplifying the ~600 bp region of 16S rRNA genes. HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) was used for PCR under the following conditions: 94 °C for 3 min followed by 32 cycles of 94 °C for 30 s; 60 °C for 40 s and 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. As described by Acosta-Martínez et al. (2008), secondary PCR (6 cycles rather than 32) was performed for FLX Amplicon Sequencing under the same condition by using designed special fusion primers with different tag sequences as: LinkerA-Tags-530F and LinkerB-1100R. After secondary PCR, all amplicon products from different samples were mixed in equal volumes, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA).

2.5. Massively parallel pyrosequencing, tag design and sequence processing

Moderate diversity pyrosequencing analysis (2000–3000 reads per sample) was performed at the Research and Testing Laboratory (Lubbock, TX, USA). In preparation for FLX sequencing (Roche, Nutley, NJ, USA), the size and concentration of DNA fragments were accurately measured using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner Biosystems, CA, USA). A 9.6 × 10⁶ sample of double-stranded DNA molecules/µl with a size of 625 bp were combined with 9.6 million DNA capture beads, and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a 70 × 75 GS PicoTiterPlate (PTP) by using a Genome Sequencer FLX System (Roche, Nutley, NJ, USA). It should be noted that 100 total samples were run within this same FLX 2-region sequencing reaction. Forty-two samples were analyzed for this project and the additional 58 tagged samples were associated with unrelated studies. All FLX related procedures were performed following Genome Sequencer FLX System manufacturers instructions (Roche, Nutley, NJ, USA).

A custom script written in the C# within the Microsoft®.NET (Microsoft Corp, Seattle, WA, USA) development environment was utilized to generate all possible combinations of 10-mer oligonucleotide tags with GC% between 40 and 60% (Acosta-Martínez et al., 2008). From this pool, we then chose 42 individual tags with which to label our samples. Custom software developed within the Microsoft®.NET (Microsoft Corp, Seattle, WA, USA) environment was utilized for all post-sequencing processing (Dowd et al., 2005; Acosta-Martínez et al., 2008). Discussion of software code is outside the scope of this report, however, a description of the

algorithm follows. Quality trimmed sequences obtained from the FLX sequencing run were processed using a custom scripted bioinformatics pipeline as depicted in Acosta-Martínez et al. (2008). In short, each sequence was trimmed back to utilize only high quality sequence information, tags were extracted from the FLX generated multi-FASTA file, while being parsed into individual sample specific files based upon the tag sequence. Tags which did not have 100% homology to the original sample tag designation were not considered as they might be suspect in quality. Sequences which were less than 200 bp after quality trimming were not considered. Samples were then depleted of definite chimeras using B2C2 software which is described and freely available from Research and Testing Laboratory (Lubbock, TX, USA; www.researchandtesting.com/B2C2.html). The resulting sequences were then evaluated using BLASTn (Altschul et al., 1990) against a custom database derived from the RDP-II database (Maidak et al., 2001) and GenBank (<http://ncbi.nlm.nih.gov>). The sequences contained within the curated 16S database were those considered of high quality based upon RDP-II (Cole et al., 2007) standards and which had complete taxonomic information within their annotations. Identification at the species level for the purpose of this study are considered tentative. Following best-hit processing a secondary post-processing algorithm was utilized to combine genus and other taxonomic designations generating compiled data with relative abundance of each taxonomic entity within the given sample. Phylogenetic assignments were based upon NCBI taxonomic designations.

2.6. Diversity and richness analysis

Multiple sequence alignment was performed using MUSCLE (with parameter -maxiters 1, -diags1 and -sv) (Edgar, 2004). Based on the alignment, a distance matrix was constructed using DNAdist from PHYLIP version 3.6 with default parameters from Felsenstein (1989, 2005). These pairwise distances served as input to DOTUR (Schloss and Handelsman, 2005) for clustering the sequences into OTUs of defined sequence similarity that ranged from 0 to 20% dissimilarity. Based upon the literature, we can expect that 0% dissimilarity in sequences will provide dramatic overestimation of the species present in a sample, based upon rarefaction (Roesch et al., 2007). At 3% dissimilarity, we expect that we can obtain accurate estimation of the majority of species present in a given sample. The clusters based upon dissimilarity of 3%, served as OTUs for generating predictive rarefaction models and for making calculations with the richness (diversity) indexes Ace and Chao1 (Chao and Bunge, 2002) in DOTUR.

2.7. Statistical analysis

Data were analyzed with two statistical models using SAS (version 9.1.3). In the first analysis, we tested for differences among four treatments such as continuous cotton (system 1), and the grazed areas of pasture, Rye-Cotton-Wheat-Fallow, and Wheat-Fallow-Rye-Cotton (system 2). Significant differences between the two systems were evaluated with a contrast comparison. Data collected at two depths were included as a subplot factor in a split plot analysis. A mixed model for a randomized block design was used with blocks as a random effect and treatment and depth as fixed effects. When treatments (or systems) interacted with depth, we tested the simple main effect of treatment (or system) within each depth; significant simple main effect tests were followed by simple effect tests using an LSD test (Kirk, 1995). Similarly, depths were compared within treatments (or systems) when these effects interacted. Individual comparisons were made with a protected LSD test when indicated by simple main effect or main effect tests. The Shapiro-Wilk test (1965) was used to assess normality of

experimental errors (in both the main plot and subplot error terms). Mauchly's test (1940) was used for sphericity in the main plot portion of the analysis; sphericity did not apply to the subplot portion because there were only two levels of depth.

A second analysis involved only the integrated livestock-cotton system (i.e., pasture, Rye-Cotton-Wheat-Fallow, and Wheat-Fallow-Rye-Cotton) to test grazing effects (grazed and non-grazed areas). Because each treatment supported grazed and non-grazed areas, the grazing effect was considered a subplot factor in a split plot arrangement with treatment as the main plot factor. The effect of soil depth was regarded as a sub-subplot factor. A mixed model for a randomized block was used with blocks as a random effect and treatment, grazing and depth as fixed effects. Significant interactions were followed by simple main effect, and then simple effect tests as appropriate; normality and sphericity were tested with the Shapiro-Wilk (1965) and Mauchly (1940) tests.

Double dendrograms were performed using comparative functions and multivariate hierarchical clustering methods of NCSS 2007 (NCSS, Kaysville, UT) based upon the top 40 most abundant bacterial classes including the weighted-pair group clustering method and the Manhattan distance method with no scaling. Manhattan distance for the relative percentage data is calculated between rows j and k , where $\delta_{ijk} = z_{ij} - z_{jk}$:

$$d_{jk} = \frac{\sum_{i=1}^p |\delta_{ijk}|}{p}$$

It should be noted that the dendrogram linkages of the bacterial classes are not phylogenetic but based upon abundance of classes among the samples ordered in rows. Clustering of the systems was similarly based upon abundance of the top 40 most abundant bacterial classes among individual samples.

Canonical correspondence analysis (CCA) was used to investigate patterns of soil bacterial occurrence in the systems and to explore the relationship of soil bacteria and other soil properties such as soil water, soil pH, MBC, MBN, total C and total N (Ter Braak, 1986). Ordination plots of the results from CCA were performed for each soil sampling depth using program cca in Oksanen et al. (2009).

3. Results

3.1. Selected properties and bacterial phyla distribution in this soil

Most soil properties listed in Table 1 were generally higher at 0–5 cm compared to 5–15 cm in the integrated livestock-cotton system whereas most soil properties (i.e., total C, MBC, MBN and enzyme activities) did not differ with soil depth in the continuous cotton system, except for bacterial FAME indicators (G- and actinomycetes) and soil moisture. At 0–5 cm, higher ($P < 0.05$) total C and MBC contents were detected in soil under pasture and the rotation within the integrated livestock-crop system compared to continuous cotton. The trend was different for soil MBN among treatments, but the mean of the integrated livestock-cotton system was significantly higher compared to the continuous cotton system. The relative abundance of bacterial populations according to FAME indicators showed this trend: continuous cotton = Rye-Cotton-Wheat-Fallow > Wheat-Fallow-Rye-Cotton > pasture. The FAME indicators for fungal populations showed this trend: pasture > Wheat-Fallow-Rye-Cotton > Rye-Cotton-Wheat-Fallow = continuous cotton. The activities of β -glucosidase, β -glucosaminidase and alkaline phosphatase showed a significantly higher mean value in soil under the integrated livestock-cotton system (pasture and the rotation) compared to continuous cotton. At 5–15 cm, the mean for soil total C was significantly higher in the integrated livestock-cotton system compared to the continuous cotton system. There was no differentiation between the treatments

Table 1

Selected soil properties of the systems studied at 0–5 and 5–15 cm.

Soil properties	Depth	System 1	Integrated livestock-cotton system (grazed areas)			System 2
		Continuous cotton	Pasture	Rye-Cotton-Wheat-Fallow	Wheat-Fallow-Rye-Cotton	Mean
Total C (g kg ⁻¹ soil)	0–5 cm	11.4 c ^a A ^b X ^c	16.20 b A	19.50 a A	14.90 b A	16.90 A Y
	5–15 cm	10.0 b A X	11.40 ab B	12.60 a B	10.20 b B	11.40 B Y
Total N (g kg ⁻¹ soil)	0–5 cm	1.1	1.3	1.7	1.6	1.5
	5–15 cm	0.8	0.5	1.0	1.0	0.9
	Mean	0.9 b X ^d	0.9 b	1.4 a	1.3 a	1.2 Y
MBC (mg C kg ⁻¹ soil)	0–5 cm	157.27 c A X	292.07 b A	252.90 b A	360.86 a A	301.95 A Y
	5–15 cm	147.65 a A X	148.96 a B	175.18 a B	175.91 a B	166.68 B X
MBN (mg N kg ⁻¹ soil)	0–5 cm	12.09 b A X	18.26 b A	16.90 b A	35.03 a A	23.40 A Y
	5–15 cm	17.14 a A X	7.63 b B	20.76 a A	22.79 a B	17.06 B X
DNA (ng mg ⁻¹ soil)	0–5 cm	3.35	4.76	3.54	3.24	3.84
	5–15 cm	2.82	3.85	3.31	2.83	3.33
	Mean	3.09 b X	4.31 a	3.43 b	3.04 b	3.59 Y
Bacterial populations (%)	0–5 cm	16.13 a A X	7.32 c A	15.23 a A	12.32 b A	11.63 A Y
	5–15 cm	17.87 a B X	5.03 c B	16.12 a A	13.48 b A	11.55 A Y
G+ populations (%)	0–5 cm	11.41 a A	4.45 b A	10.27 a A	10.54 a A	8.42
	5–15 cm	11.95 a A	3.38 c A	10.78 a A	9.02 b B	7.73
	Mean	11.68 X	3.92	10.53	9.78	8.08 Y
G– populations (%)	0–5 cm	3.01 a A	1.26 b A	1.78 b A	1.47 b A	1.51
	5–15 cm	1.83 a B	0.41 b B	1.53 a A	1.13 ab A	1.03
	Mean	2.42 X	0.84	1.66	1.31	1.27 Y
Actinomycetes (%)	0–5 cm	1.71 b A	1.60 b A	3.17 a A	0.30 c A	1.69
	5–15 cm	4.08 a B	1.23 b A	3.80 a A	3.32 a B	2.78
	Mean	2.90 X	1.42	3.49	1.82	2.24 Y
Fungal populations (%)	0–5 cm	17.07 c A X	41.00 a A	16.77 c A	21.67 b A	26.48 A Y
	5–15 cm	17.86 c A X	50.46 a B	23.91 b B	26.44 b B	33.60 B Y
% Moisture	0–5 cm	16.83 a A X	14.15 b A	16.57 a A	17.40 a A	16.04 X
	5–15 cm	22.10 a B X	13.68 c A	20.17 ab B	19.35 b B	17.71 Y
pH	0–5 cm	7.68	7.07	7.34	7.18	7.20
	5–15 cm	7.90	7.32	7.57	7.48	7.46
	Mean	7.80 a X	7.20 b	7.46 ab	7.33 b	7.33 Y
β-Glucosaminidase activity	0–5 cm	15.68 c A X	70.23 a A	27.38 b A	31.61 b A	43.07 A Y
	5–15 cm	12.97 b A X	23.45 a B	11.87 c B	16.26 b B	17.19 B Y
β-Glucosidase activity	0–5 cm	82.92 c A X	295.58 a A	181.31 b A	323.42 a A	266.77 A Y
	5–15 cm	63.36 b A X	106.52 a B	56.72 b B	91.28 ab B	84.84 B X
Alkaline phosphatase act.	0–5 cm	125.80 c A X	214.10 b A	247.48 a b A	272.98 a A	244.86 A Y
	5–15 cm	118.85 a A X	149.32 a A	150.54 a B	168.71 a B	156.19 B Y

^a Treatment (continuous cotton, pasture, rye-cotton-wheat-fallow, wheat-fallow-rye-cotton) means within a depth followed by the same lower case letter (a, b and c) are not significantly different ($P > 0.05$).

^b Depth means within a column followed by the same upper case letter (A and B) are not significantly different ($P > 0.05$).

^c System means within a depth followed by the same upper case letter (X and Y) are not significantly different ($P > 0.05$).

^d When there are no differences with soil depth for a treatment, a mean for the two depths was calculated which if followed by the same lower case letter (a, b and c) or capital letter (X and Y) are not significantly different ($P > 0.05$) for treatment or system effects.

in their soil MBC and MBN at this lower soil depth. The mean of the FAME indicators for bacterial populations (G+, G– and actinomycetes) in the integrated livestock-cotton system was significantly different (lower) compared to the continuous cotton system. The opposite trend was found for the fungal FAMES, which were generally highest under pasture compared to the other treatments. Higher enzyme activities were found at this lower soil depth under the integrated livestock-cotton system compared to the continuous cotton system, except for β-glucosidase activity.

Fig. 2 provides a hierarchical clustering double dendrogram based upon the relative percentage of the top 40 bacteria (Y-axis) including each treatment (continuous cotton, Rye-Cotton-Wheat-Fallow, Wheat-Fallow-Rye-Cotton and pasture), system (integrated livestock-cotton system and continuous cotton system) and soil depth (0–5 and 5–15 cm) evaluated in our study (X-axis). Thus, this type of dendrogram enables us to visualize the entire dataset. The heatmap colors indicate the relative percentage of bacteria ranging from <0.0001% in black up to 65% in red. To highlight key trends, it

is possible to visualize that the predominant phyla at 0–5 and 5–15 cm in this soil (up to 65% of abundance) despite system effects were *Proteobacteria*, *Actinobacteria* and *Gemmatimonadetes*. Less predominant bacteria in this soil (3–8%) were *Acidobacteria*, *Verrucomicrobiae*, *Thermomicrobia*, *Spartobacteria*, *Solibacteres*, *Nitrospira*, *Spirochetes*, *Chloroflexi* and *Planctomycetacia*. Definitely rare bacteria, but still detected in this soil (<1.03%) included *Globobacteria*, *Thermolithobacteria*, *ε-Proteobacteria*, *Erysipelotrichi*, *Fribrobacteres*, *Thermotogae*, *Aquificae*, *Chroobacteria*, *Mollicutes*, *Fusobacteria*, *Ktedonobacteria* and *Dehalococcoidetes*.

3.2. Bacterial phyla distribution as affected by soil depth, systems, and grazing

The distribution of bacterial phyla was not different at 0–5 and 5–15 cm depths under continuous cotton, but *Proteobacteria*, *Firmicutes* and *Chloroflexi* differed with soil depth within the integrated livestock-cotton system (Table 2). The first

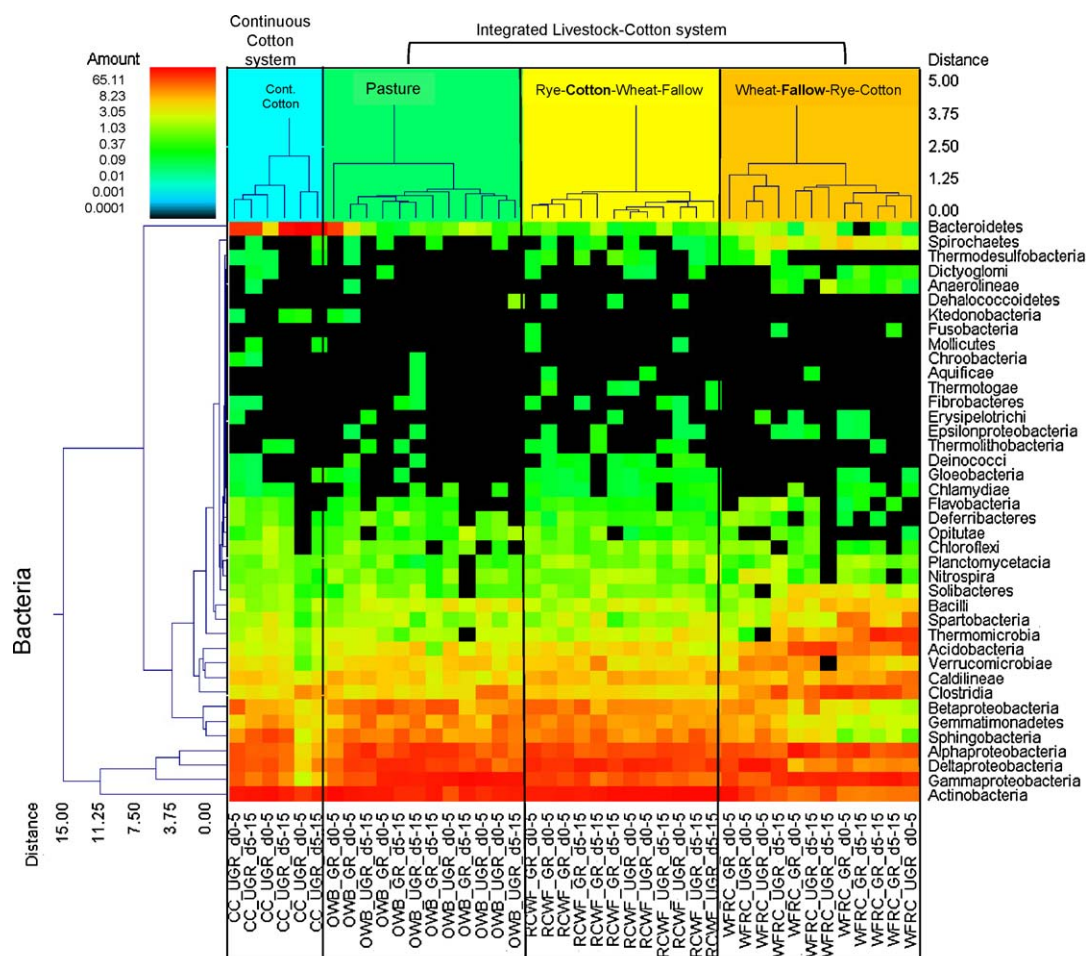


Fig. 2. Double hierarchal dendrogram to evaluate bacteria distribution in this soil as affected by the treatments using the weighted-pair group clustering method and Manhattan distance method with no scaling. It should be noted that the dendrogram linkages of the bacterial classes are not phylogenetic. The heat map depicts the relative percentage of each bacteria (variables clustering on Y-axis) within each treatment (X-axis clustering). Clustering of the treatments was similarly based upon abundance of the top 40 most abundant bacterial classes among these treatments. The X-axis at the top shows the four treatments (continuous cotton, Wheat-Fallow-Rye-Cotton, Rye-Cotton-Wheat-Fallow and pasture) while a more detailed explanation per sample is given at the bottom of this axis (CC = continuous cotton, OWB = pasture, RCWF = Rye-Cotton-Wheat-Fallow, WFRC = Wheat-Fallow-Rye-Cotton, GR = grazed, UGR = non-grazed, d0–5 = 0–5 cm, and d5–15 = 5–15 cm). The heat map colors represent the relative percentage of the bacterial classes within each treatment with the legend indicated at the upper left of the figure. The treatments along the X-axis with Manhattan distances are indicated by branch length and an associated scale located at the upper right of the figure. Clustering based upon Manhattan distance of the bacterial classes along the Y-axis and their associated scale is indicated in the lower left.

distinct trend found from this study was the higher predominance of *Proteobacteria* under the integrated livestock-cotton system (pasture and rotation) compared to continuous cotton at 0–5 and 5–15 cm depths. The second distinct trend found was the predominance of *Bacteroidetes* in soil under the continuous cotton system compared to the integrated livestock-cotton system. There were also differences in bacterial phyla distribution in the rotation when sampled under fallow periods or cotton. For example, *Firmicutes* (i.e., *Clostridia*) and *Chloroflexi* (i.e., *Thermomicrobia*) were more abundant when the rotation was sampled under fallow than under cotton at 0–5 and 5–15 cm (some data not shown).

In the integrated livestock-cotton system, comparison of soil bacteria under grazed areas vs. non-grazed areas revealed examples of bacteria that are involved in a two-way interaction of treatment and grazing effects (data not shown). For example, *Gemmatimonadetes* were higher under non-grazed areas than grazed areas of pasture, but this trend was not detected in the rotation. Other bacteria such as *Actinobacteria*, *Proteobacteria* and *Chloroflexi* were affected by a three-way interaction between grazing, treatment and soil depth (Table 3), which makes it

difficult to establish the effects of grazing on bacterial phyla distribution. For example, γ -*Proteobacteria* were higher in the non-grazed areas than grazed areas of pasture at 0–5 cm and the opposite trend was detected at 5–15 cm while these trends were not apparent in the rotation (sampled under cotton or fallow). In addition, *Actinobacteria* were lower in the non-grazed areas than grazed areas of pasture at 0–5 cm only, while the opposite trend was detected for the rotation under cotton (Rye-Cotton-Wheat-Fallow) at both soil depths (0–5 and 5–15 cm).

In order to better visualize the patterns of occurrence and abundance of the predominant bacterial phyla of this soil as affected by management, the integrated livestock-cotton system (grazed and non-grazed areas) and continuous cotton system were compared using the double dendrogram shown in Fig. 2. This dendrogram further showed that soil under continuous cotton had high percentages of *Bacteroidetes* compared to the integrated livestock-cotton system. In addition, soil from the rotation sampled under fallow (Wheat-Fallow-Rye-Cotton) had less *Actinobacteria*, *Sphingobacteria*, *Gemmatimonadetes*, and β -*Proteobacteria* whereas higher *Verrucomicrobiae*, *Acidobacteria* and *Thermomicrobia* compared to the other treatments.

Table 2

Relative abundance of bacteria in the integrated livestock-cotton system compared to continuous cotton.

Bacteria	Depth	System 1	Integrated livestock-cotton system (grazed areas)			System 2	Depth mean
		Continuous cotton	Pasture	Rye-Cotton-Wheat-Fallow	Wheat-Fallow-Rye-Cotton	Mean	
Relative abundance (%)							
<i>Proteobacteria</i>	0–5 cm	27.32 b ^a A ^b	41.00 a A	46.15 a A	41.66 a A	42.93	39.03
	5–15 cm	24.08 c A	56.32 a B	42.63 b A	38.73 b A	45.89	40.44
	Mean	25.70 X ^c	48.66	44.39	40.00	44.41 Y	
<i>Bacteroidetes</i>	0–5 cm	33.31	12.34	7.76	2.80	7.63	14.05 A
	5–15 cm	37.52	8.49	7.68	1.21	5.79	13.72 A
	Mean	35.41 a X	10.41 b	7.71 b	2.00 c	6.71 Y	
<i>Firmicutes</i>	0–5 cm	4.13 b A	3.56 b A	4.04 b A	9.97 a A	5.86	5.43
	5–15 cm	4.00 b A	2.97 b A	3.93 b A	15.09 a B	7.33	6.50
	Mean	4.06 X	3.27	3.99	12.53	6.59 Y	
<i>Chloroflexi</i>	0–5 cm	4.27 b A	5.49 b A	5.91 b A	8.87 a A	6.76	6.14
	5–15 cm	4.49 b A	2.88 b A	5.54 b A	18.23 a B	8.88	7.79
	Mean	4.38 X	4.19	5.73	13.55	7.82 Y	
<i>Verrucomicrobiae/Chlamydiae</i>	0–5 cm	3.10	3.59	4.86	7.54	5.33	4.77 A
	5–15 cm	2.75	4.54	5.26	7.36	5.72	4.97 A
	Mean	2.92 c X	4.06 b c	5.06 b	7.45 a	5.53 Y	
<i>Gemmatimonadetes</i>	0–5 cm	3.83	4.23	6.91	2.46	4.53	4.36 A
	5–15 cm	3.73	4.85	6.13	1.23	4.07	3.99 A
	Mean	3.78 b X	4.54 b	6.52 a	1.85 c	4.30 X	
<i>Acidobacteria/Fibrobacteres</i>	0–5 cm	1.68	2.31	2.89	8.66	4.62	3.88 A
	5–15 cm	1.70	1.48	3.45	8.12	4.35	3.69 A
	Mean	1.69 b X	1.89 b	3.17 b	8.39 a	4.49 Y	
<i>Spirochaetes</i>	0–5 cm	0.00	0.04	0.02	1.49	0.52	0.38 A
	5–15 cm	0.08	0.04	0.09	1.76	0.63	0.49 A
	Mean	0.04 b X	0.04 b	0.05 b	1.63 a	0.57 Y	
<i>Planctomycetacia</i>	0–5 cm	0.52	0.61	0.71	0.47	0.60	0.58 A
	5–15 cm	0.42	0.08	0.49	0.61	0.39	0.40 A
	Mean	0.47 a X	0.35 a	0.60 a	0.54 a	0.49 X	

^a Treatment (continuous cotton, pasture, Rye-Cotton-Wheat-Fallow, Wheat-Fallow-Rye-Cotton) means within a depth (a, b and c) are not significantly different ($P > 0.05$).^b Depth means within a column followed by the same upper case letter (A and B) are not significantly different ($P > 0.05$).^c System means within a depth followed by the same upper case letter (X and Y) are not significantly different ($P > 0.05$).

3.3. Bacterial diversity indexes affected by soil depth, systems, and grazing

The maximum OTUs in this soil according to rarefaction curves at 3% dissimilarity were 1200 and 1100 at 0–5 and 5–15 cm depths, respectively (Fig. 3). A distinctive trend from the rarefaction curves was the lowest number of OTUs in the rotation sampled under fallow periods (Wheat-Fallow-Rye-Cotton) compared to the other

systems sampled under cotton (Rye-Cotton-Wheat-Fallow or continuous cotton) or grass vegetation (pasture). The different bacterial diversity indexes used (Ace, Chao1, and OTU) did not show consistent trends between the integrated livestock-cotton system and continuous cotton as other soil properties, but higher bacterial diversity indexes were found at 0–5 than 5–15 cm depth (Table 4). Within the integrated livestock-cotton system, the most distinct trend found was the higher bacterial diversity indexes in

Table 3

Soil bacteria affected by a three-way interaction between grazing, treatment and depth within the integrated livestock-cotton system.

Bacteria	Pasture		Rye-Cotton-Wheat-Fallow		Wheat-Fallow-Rye-Cotton	
	Grazed	Non-Grazed	Grazed	Non-Grazed	Grazed	Non-Grazed
0–5 cm						
<i>Proteobacteria</i>	41.00 a ^a A ^b X ^c	53.76 a A Y	46.15 a A X	40.87 b A X	41.66 aAX	42.61 b A X
<i>γ-Proteobacteria</i>	11.40 a AX	25.89 a AY	11.56 a AX	10.11 b AX	15.48 a AX	11.17 b AX
<i>Actinobacteria</i>	25.92 a A X	13.49 b A Y	19.64 b A X	29.58 a A Y	15.35 b A X	10.90 b A X
<i>Thermomicrobia</i>	1.11 a A X	1.03 a A X	0.98 a A X	1.70 a A X	3.82 b A X	4.59 b A X
<i>Solibacteres</i>	0.41 b A X	0.29 a A X	0.56 b A X	0.63 a A X	1.96aAX	0.52 a A Y
<i>Chloroflexi</i>	5.49 a A X	3.67 a A X	5.92 a A X	6.67 a A X	8.87aAX	10.56 b A X
5–15 cm						
<i>Proteobacteria</i>	56.32 a B X	48.94 a A X	42.63 b A X	36.56 b A X	38.74 b A X	40.17 b A X
<i>γ-Proteobacteria</i>	24.80 a BX	17.15 a BY	10.14 c AX	7.74 b AX	20.08 b BX	15.35 a AY
<i>Actinobacteria</i>	17.84 b B X	17.84 b A X	23.41a A X	32.41 a A Y	7.10 c B X	9.62 c A X
<i>Thermomicrobia</i>	0.39a A X	1.16a A X	1.19 a A X	1.56 b A X	12.44 b B X	3.54 c A Y
<i>Solibacteres</i>	0.18 c A X	0.78 a B Y	0.68 b A X	0.44 a A X	1.55 a A X	1.89 b B X
<i>Chloroflexi</i>	2.88 a A X	4.84aAX	5.54 a A X	6.12 a A X	18.23 b B X	7.69 a A Y

^a Treatment (pasture, Rye-Cotton-Wheat-Fallow, Wheat-Fallow-Rye-Cotton) means within level of grazing and a depth followed by the same lower case letter (a, b and c) are not significantly different ($P > 0.05$).^b Depth means within a treatment and a level of grazing followed by the same upper case letter (A and B) are not significantly different ($P > 0.05$).^c Grazed/non-grazed means within a treatment and a depth followed by the same upper case letter (X and Y) are not significantly different ($P > 0.05$).

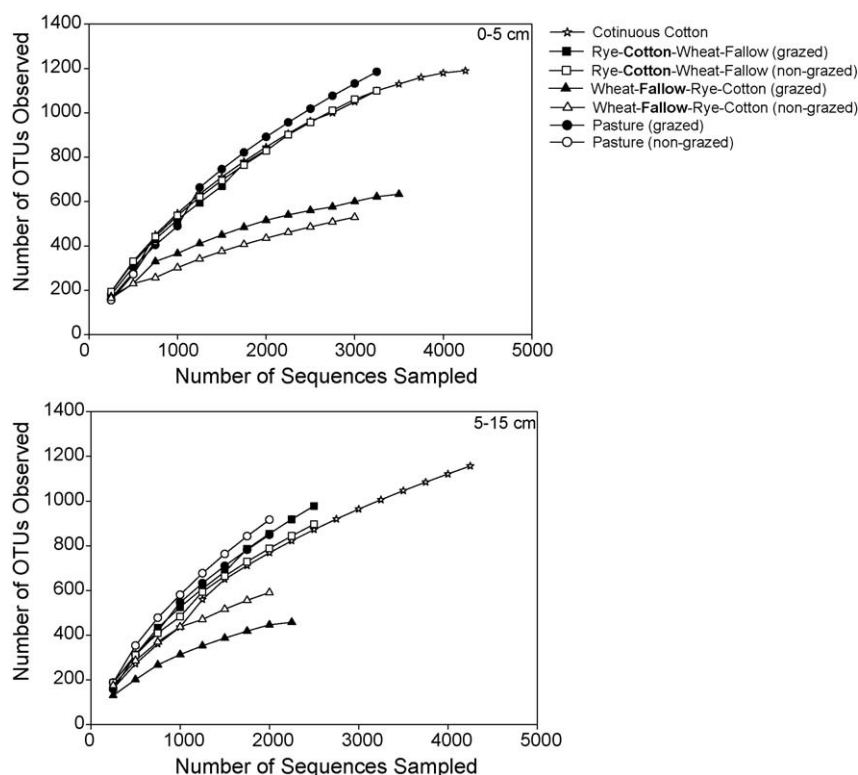


Fig. 3. Rarefaction curves depicting the effect of 3% dissimilarity on the number of OTUs identified in this soil at 0–5 and 5–15 cm depths.

Table 4

Soil bacterial diversity indexes as affected by the integrated livestock-cotton system and continuous cotton system.

Bacterial diversity index	Soil depth (cm)	System 1	Integrated livestock-cotton system (grazed areas)			System 2	Depth mean
		Continuous cotton	Pasture	Rye-Cotton-Wheat-Fallow	Wheat-Fallow-Rye-Cotton	Mean	Across treatments
Ace (3%)	0–5	1737.67	1576.00	1462.33	1046.00	1361.44	1455.50 A ^a
	5–15	1440.00	1084.67	1505.00	648.00	1079.22	1169.42 B
	Mean	1588.83 a ^b X ^c	1330.33 a	1483.67 a	847.00 b	1220.33 Y	
Chao1 (3%)	0–5	1660.67	1548.67	1329.00	1038.33	1305.33	1394.17 A
	5–15	1327.67	1037.00	1394.67	640.44	1024.00	1099.92 B
	Mean	1494.17 a X	1292.83 a	1361.83 a	839.33 b	1164.67 Y	
OTU (3%)	0–5	836.67	733.00	731.00	562.67	675.56	715.83 A
	5–15	707.33	460.00	669.67	380.00	503.22	554.25 A
	Mean	772.00 a X	596.50 bc	700.00 ab	460.00 c	589.39 X	

^a Depth means within a column followed by the same upper case letter (A and B) are not significantly different ($P > 0.05$).

^b Treatment (continuous cotton, pasture, Rye-Cotton-Wheat-Fallow, Wheat-Fallow-Rye-Cotton) means within a depth followed by the same lower case letter (a, b and c) are not significantly different ($P > 0.05$).

^c System means within a depth followed by the same upper case letter (X and Y) are not significantly different ($P > 0.05$).

Table 5

Soil bacterial diversity indexes as affected by grazing within the integrated livestock-cotton system.

Bacterial diversity indexes	Pasture		Rye-Cotton-Wheat-Fallow		Wheat-Fallow-Rye-Cotton	
	Grazed	Non-Grazed	Grazed	Non-Grazed	Grazed	Non-Grazed
0–5 cm						
Ace	1567.00 a ^a A ^b X ^c	834.33 b A Y	1462.33 a A X	2095.00 a A Y	1046.00 a A X	902.33 b A X
Chao1	1548.67 a A X	783.67 b A Y	1329.00 a A X	2033.33 a A Y	1038.33 a A X	852.67 b A X
5–15 cm						
Ace	1084.67 ab B X	1478.67 a B X	1505.00 a A X	1368.33 a B X	648.00 b A X	705.33 b A X
Chao1	1037.00 ab B X	1347.00 a B X	1394.67 a A X	1363.00 a B X	640.33 b A X	683.00 b A X

^a Treatment (pasture, Rye-Cotton-Wheat-Fallow, Wheat-Fallow-Rye-Cotton) means within a level of grazing and depth followed by the same lower case letter (a and b) are not significantly different ($P > 0.05$).

^b Depth means within a treatment and a level of grazing followed by the same upper case letter (A and B) are not significantly different ($P > 0.05$).

^c Grazed/non-grazed means within a treatment and a depth followed by the same upper case letter (X and Y) are not significantly different ($P > 0.05$).

Table 6

Correlations among selected bacteria and soil properties.

Soil property	<i>Proteobacteria</i>	<i>Actinobacteria</i>	<i>Bacteroidetes</i>	<i>Firmicutes</i>	<i>Chloroflexi</i>	<i>Verrucomicrobiae/Chlamydiae</i>	<i>Fibrobacteres</i>	<i>Nitrospira</i>	<i>Spirochaetes</i>	<i>Planctomycetacia</i>
0–5 cm										
MBC (mg kg ⁻¹)	0.481 [*]		−0.715 ^{***}	0.475 [*]	0.471 [*]	0.636 ^{**}	0.541 ^{**}		0.455 [*]	
MBN (mg kg ⁻¹)			−0.475 [*]	0.468 [*]	0.437 [*]	0.642 ^{**}	0.514 [*]	0.455 [*]	0.544 ^{**}	
Total C (g kg ⁻¹)	0.634 ^{**}		−0.693 ^{***}							
Total N (g kg ⁻¹)	0.571 ^{**}		−0.681 ^{***}		0.466 [*]	0.431 [*]	0.467 [*]			
pH										
Bacterial FAMES	−0.547 ^{**}									
G+ (%)	−0.485 [*]									
G− (%)	−0.779 ^{***}		0.841 ^{***}							
Actinomycetes (%)		0.510 [*]		−0.550 ^{**}		−0.602 ^{**}	−0.514 [*]		−0.573 ^{**}	
β-glucosaminidase act.	0.618 ^{**}									
β-glucosidase act.	0.601 ^{**}		−0.703 ^{***}	0.523 [*]		0.500 [*]	0.480 [*]			
Alkaline phosphatase act.	0.649 ^{**}	−0.518 [*]	−0.843 ^{***}	0.592 ^{**}	0.434 [*]	0.575 ^{**}	0.479 [*]			
5–15 cm										
MBC (mg kg ⁻¹)										
MBN (mg kg ⁻¹)				0.525 ^{**}	0.445 [*]		0.606 ^{**}		0.552 ^{**}	
Total C (g kg ⁻¹)			−0.438 [*]							
Total N (g kg ⁻¹)										
pH	−0.578 ^{**}		0.529 [*]							
Bacterial FAMES	−0.769 ^{***}									0.507 [*]
G+ (%)	−0.762 ^{***}									0.468 [*]
G− (%)	−0.613 ^{**}							0.452 [*]		0.536 ^{**}
Actinomycetes (%)	−0.750 ^{***}									0.521 [*]
β-glucosaminidase act.	0.512 [*]									
β-glucosidase act.	0.463 [*]									
Alkaline phosphatase act.		−0.454 [*]	−0.582 ^{**}				0.481 [*]			

^{*} $P < 0.05$.^{**} $P < 0.01$.^{***} $P < 0.001$.

grazed areas of pasture compared to the non-grazed areas and under non-grazed than grazed areas of the rotation sampled under cotton (Rye-Cotton-Wheat-Fallow) at 0–5 cm (Table 5).

3.4. Correlations among selected soil properties and bacterial phyla distribution

Linear regression analyses showed that most significant correlations were found at 0–5 cm between bacterial phyla and selected soil properties such as total C, total N, MBC, MBN, pH and bacterial FAMEs (Table 6). For example, *Proteobacteria* showed a positive correlation with MBC, total C, total N and enzyme activities, but a negative correlation with FAME indicators for G– populations. *Actinobacteria* showed only a positive correlation with FAME indicators for actinomycetes. *Bacteroidetes* showed a significant negative correlation with MBC, MBN, total C, total N and the activities of β -glucosidase and alkaline phosphatase, whereas it showed a positive correlation with FAME indicators for G– populations. *Firmicutes*, *Verrucomicrobiae*, *Spirochaetes* and *Chloroflexi* showed a positive correlation with MBC, MBN and with at least one of the enzyme activities studied. *Chloroflexi* and *Verrucomicrobiae* showed a positive correlation with total N. At 5–15 cm, *Proteobacteria* showed a negative correlation with pH, the bacterial FAME groups (G+, G– and actinomycetes) and the activities of β -glucosidase and alkaline phosphatase. *Firmicutes* showed a positive correlation with MBN. *Fibrobacteres* showed a positive correlation with MBC and alkaline phosphatase activity.

The correlations between soil bacteria and other soil properties (MBC, MBN, total C, total N, soil moisture and soil pH) were also evaluated using ordination plots according to the CCA. The ordination plots along axes 1 and 2 showed an arch effect which suggests that axis 2 is related to axis 1; thus, our ordination plots will show axes 1 and 3 (Fig. 4). The first axis for both soil depths was related to variation in bacterial populations associated with continuous cotton (plotted on the far positive side of axis 1) and the systems under study (plotted nearer the origin and on the negative end of axis 1). At 0–5 cm, MBC, MBN and soil pH (and to a less extent, total C and total N) were important variables associated with the first axis. *Bacteroidetes* were centered along a gradient with relatively high soil pH and relatively low values of MBC and MBN. In contrast, *Spirochaetes* and *Acidobacteria/Fibrobacteres* were associated with low soil pH and higher values of MBC and MBN. Some of the trends revealed by the ordination plots (Fig. 4) were only partially revealed by the linear regression analyses (Table 6). For example, *Bacteroidetes* and *Spirochaetes* were correlated (in different directions) with MBC and MBN (Table 6) and this is reflected in our ordination plots (Fig. 4). Additionally, *Bacteroidetes* were negatively correlated with soil total C and total N, a trend that is apparent in Fig. 4 and Table 6. However, none of these bacterial phyla were correlated with soil pH in Table 6 whereas our ordination plots (Fig. 4) show that soil pH was an important variable associated with axis 1. The second major source of variation (expressed along axis 3) at 0–5 cm largely reflects variation among the systems. For example, non-grazed areas in pasture were plotted on the far positive end of axis 3 whereas grazed areas (as well as the remaining treatments) were plotted nearer the origin and on the negative end of this axis. The rotation (Rye-Cotton-Wheat-Fallow and Wheat-Fallow-Cotton-Rye) was also arranged along axis 3. However, in contrast to pasture, grazed areas of the rotation (Rye-Cotton-Wheat-Fallow and Wheat-Fallow-Cotton-Rye) were assigned more positive scores on the third axis than their non-grazed counterparts. The third axis was correlated with soil moisture content. *Nitrospira* (class) and *Chloroflexi* were centered on areas with higher soil moisture content whereas *Firmicutes* were centered on areas with lower soil moisture content.

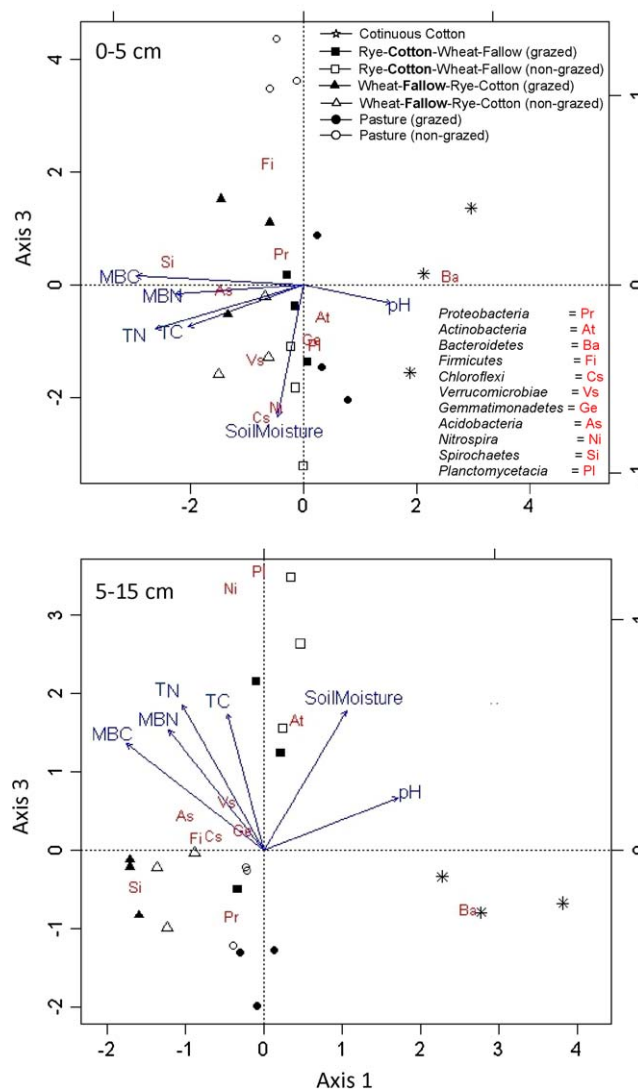


Fig. 4. Ordination plots of the results from canonical correspondence analysis (CCA) to explore the relationship between bacterial populations (shown in red) and selected soil properties such as total C, total N, soil moisture, MBC, MBN and soil pH (vectors) in the continuous cotton system and the grazed and non-grazed areas of the integrated livestock-cotton system (pasture, Wheat-Fallow-Rye-Cotton and Rye-Cotton-Wheat-Fallow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The ordination plot for 5–15 cm depth also indicated that patterns in soil bacteria were related to differences between continuous cotton (plotted on the far positive end of axis 1) and the integrated livestock-cotton system. As at the 0–5 cm depth, *Bacteroidetes* were associated with relatively high soil pH values and *Spirochaetes* were associated with relatively low soil pH. The second major direction of variation (plotted along axis 3) generally expressed differences between Rye-Cotton-Wheat-Fallow (positioned on the positive end of this axis) and other treatments. Differences related to grazed and non-grazed areas were less apparent at 5–15 cm than at 0–5 cm.

4. Discussion

4.1. Predominance of bacteria in this soil

The distribution of bacterial phyla in the soil described here agrees with results from other soils (Janssen, 2006), where the majority of 16S rRNA gene soil clone libraries belong to nine major

bacteria phyla. The most dominant bacteria in this soil were *Proteobacteria*, which is in agreement with a summary reported by Spain et al. (2009) for several soils. These authors concluded that *Proteobacteria* can represent 25–40% of total sequences by clone library studies (i.e., >1000 near full length 16S rRNA genes or >300 bp 16S rRNA gene sequences) or 42–50% abundance from shorter fragments (~100 bp) obtained by pyrosequencing. Liebner et al. (2008) summarized that, based on more than 30 clone libraries and other studies (Janssen, 2006; Dinamarca et al., 2007), α -, β - and γ -*Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and to a lesser extent *Firmicutes*, *Bacteroidetes* and *Planctomycetes* have been identified as major soil phyla, although recognizing that their relative abundances vary with the study site.

4.2. Bacterial phyla distribution as affected by the systems

Significant differences were found in soil bacterial phyla distribution 10 years after changing from a specialized continuous cotton system to an integrated livestock-cotton production system. One of the most distinctive trends was the higher *Proteobacteria* in soil under the rotation and pasture within the integrated livestock-cotton system compared to continuous cotton. This change may be associated to the higher soil enzyme activities and total C under the integrated system as a recent study stated that the *Proteobacteria* encompass an enormous level of morphological, physiological and metabolic diversity, and play a significant role in global C, N and S cycling (Kersters et al., 2006). It is difficult, however, to make comparisons with other studies because of the differences in soil properties, environmental conditions, and vegetation. However, the decreases in *Proteobacteria* under continuous cotton compared to pasture agree with the study by Jangid et al. (2008) using traditional cloning techniques in which cropland was compared to pasture. As a difference to the study of Jangid et al. (2008), our soil was characterized by very high clay and organic matter contents and soil pH.

Another distinctive trend in this study was the higher *Bacteroidetes* in soil under cropland compared to pasture, which agrees with other studies using traditional DNA cloning (Jangid et al., 2008) and pyrosequencing (Roesch et al., 2007; Acosta-Martínez et al., 2008). We attribute the predominance of *Bacteroidetes* in soil under continuous cotton to their ability to rapidly exploit bio-available organic matter and colonize aggregates which may become available as crops are planted after long winter fallow periods in this system (Weiss et al., 1996; Abell and Bowman, 2005; Acosta-Martínez et al., 2008). In addition, previous studies have discussed that this bacterial phyla is enriched with either anteiso- or unsaturated cellular fatty acids that enhance the ability of microbial cells to survive low temperatures which can occur more often to soil under continuous cotton due to the intensive tillage operations and winter fallow periods (Kaneda, 1991; Nichols et al., 1993; Weber et al., 2001; Liebner et al., 2008).

The important role of *Actinobacteria* in soil metabolic functioning is well known due to their involvement in decomposition of organic materials, such as cellulose and chitin, thereby playing a crucial role in organic matter turnover and C cycling (Lacey, 1973). A previous study with this soil in another location showed higher *Actinobacteria* as well as microbial biomass and nutrient cycling enzyme activities in non-disturbed systems under grasses (i.e., Conservation Reserve Program and pastures) compared to continuous cotton (Acosta-Martínez et al., 2008); which agree with our findings of higher *Actinobacteria* and enzyme activities of this pasture (i.e., grazed areas) compared to continuous cotton. However, there was lower abundance of *Actinobacteria* in the rotation sampled under fallow (Wheat-Fallow-Rye-Cotton) compared to the other treatments (revealed by the double dendrogram) while microbial biomass, fungal

populations and enzyme activities were still higher in this rotation compared to continuous cotton.

The higher abundance of *Chloroflexi* (i.e., *Thermobacteria*) and *Firmicutes* when sampling under fallow periods in the rotation (Wheat-Fallow-Rye-Cotton) compared to sampling under cotton (Rye-Cotton-Wheat-Fallow or continuous cotton) or grass vegetation (pasture) may be of ecological significance. This trend could emphasize the ability of these types of bacteria to survive very low substrate availability under lack of plant rhizosphere. It is also possible that these trends could emphasize the ability of these types of bacteria to survive extreme environmental changes when soil is more exposed under summer fallow periods. Within our literature review, not much information was found for the *Firmicutes* in this regard, but the few studies found about *Chloroflexi* mostly reported the occurrence of this bacterial phyla in systems experiencing extremely high temperatures including sludge (Björnssona et al., 2002).

4.3. Bacterial phyla distribution as affected by grazing within the integrated livestock-cotton system

A previous study conducted in the same systems revealed higher microbial biomass and enzyme activities involved in C, N and P cycling under non-grazed areas than grazed areas of pasture soil within the integrated livestock-cotton system (Acosta-Martínez et al., submitted for publication). Similarly, Ingram et al. (2008) reported higher microbial biomass in loamy soils under lightly grazed systems at 0–5 cm in a semiarid region, which were attributed to higher amounts of high quality substrates and soil moisture content as compared to heavily grazed areas. Therefore, it is possible that higher availability of certain substrates in the non-grazed areas compared to the grazed areas of pasture may have encouraged predominance of *Proteobacteria*. On the other hand, livestock can incorporate other nutrients from dung and urine (Bardgett et al., 1997; Tracey and Frank, 1998) in the grazed areas, which may have enhanced *Actinobacteria* and *Planctomycetacia*. For the differences in bacterial phyla distribution due to grazing in the rotation under cotton or fallow periods, we cannot provide an ecological explanation because soil organic matter content or pH should not be affected by crop or seasonal variation. In addition to certain differences in substrate quality that may be developed in soil due to grazing, our results suggest that different nutrients may become available when the rotation is under cotton following rye or under fallow following wheat. This could explain the higher abundance of *Solibacteres* under grazed compared to non-grazed areas in the rotation under fallow (Wheat-Fallow-Rye-Cotton) while higher *Actinobacteria* under non-grazed areas of the rotation when sampled under cotton (Rye-Cotton-Wheat-Fallow).

4.4. Bacterial diversity as affected by soil depth, systems and grazing

Previous studies have reported decreases in microbial diversity with increasing soil depth (Zhou et al., 2002; Neufeld and Mohn, 2005). In our study, we only found decreases with depth in the overall mean of bacterial diversity calculated for each system. It is difficult to explain why the bacterial diversity indexes did not show similar trends as total C, total N, microbial biomass, DNA concentration and enzyme activities (i.e., higher under the integrated livestock-crop system). Previous studies using FAME profiling by Acosta-Martínez et al. (submitted for publication) reported different soil microbial community structures between the systems studied with a more bacterial dominated community under continuous cotton compared to the integrated livestock-crop system (continuous cotton > rotation > pasture). We only found significantly different (higher) bacterial diversity indexes at

0–5 cm in non-grazed areas than the grazed areas of the rotation when sampled under cotton following rye (Rye-Cotton-Wheat-Fallow) which agree with the microbial biomass and enzyme activities trends (i.e., higher under non-grazed areas). It is important to recognize that Kennedy (1999) explained that a diversity index is a single value that cannot fully represent the total makeup of a community, and thus, two communities may have the same diversity index value, but one may comprise low evenness and high richness, and the other may comprise high evenness and low richness. It is also important to recognize the inherent PCR bias, which is assumed to be more pronounced if a secondary PCR is used and thus, our ongoing studies have been optimized in terms of reducing PCR cycles, allowing the elimination of the secondary PCR reaction step needed in the early stage of this methodology. It was difficult to establish a comparison of previous studies with the trends found in this study because this is the first work on the bacterial diversity in a single soil type as affected by integrated livestock-crop systems by a pyrosequencing approach. In addition, bacterial phyla distribution as affected by management and land use may depend on the soil properties, environmental conditions, and the systems itself.

4.5. Correlation between bacterial phyla distribution with other soil properties

The study of soil bacterial diversity using molecular biological techniques has expanded our understanding of major bacterial groups in soil, which contribute to essential soil processes including soil aggregation and nutrient cycling (Lynch and Bragg, 1985). However, we are only beginning to explore the management-induced shifts in soil microbial communities that may be linked to soil functioning changes. The positive correlation between *Proteobacteria* with total C, microbial biomass and enzyme activities of C and N cycling in this soil suggested that they play an important role in global C and N cycling (Kersters et al., 2006). Thus, positive correlations of other bacteria such as *Chloroflexi*, *Verrucomicrobiae* and *Fibrobacteres* with soil microbial biomass C or total N found in this study may indicate their involvement in soil C and N cycling. Although not all bacteria under *Nitrospira* are nitrate oxidizers, the positive correlation found between *Nitrospira* and microbial biomass N in this study may further support their ecological relevance to soil N cycling. Similar to our trends, negative diversity–abundance correlation between the *Bacteroidetes* and other soil bacteria phyla were found by Youssef and Elshahed (2009). *Bacteroidetes* were negatively correlated with MBC, MBN, total C and total N in this soil as they were the predominant phyla under continuous cotton, which showed lower total C, microbial biomass C and N, and enzyme activities than the integrated livestock-cotton system. Thus, *Bacteroidetes* appear to play a key role in sustaining soil processes in the continuous cotton system. Several bacterial phyla (*Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Verrucomicrobiae* and *Fibrobacteres*) were significantly ($P < 0.05$) correlated with alkaline phosphatase activity in agreement with previous statements that alkaline phosphatase is derived totally from microorganisms (Tabatabai, 1994). Similarly, several bacteria (*Proteobacteria*, *Firmicutes*, *Verrucomicrobiae*, *Fibrobacteres*) were found to be positively correlated to β -glucosidase activity (0–5 cm), which is involved in the last step of cellulose degradation in soil. However, only *Proteobacteria* were positively correlated with β -glucosaminidase activity, which is involved in chitin degradation. The lack of correlation between most bacteria with β -glucosaminidase activity may confirm that this enzyme is mainly produced by fungal populations (Parham and Deng, 2000). The soil microbial community shifts to higher fungal populations (according to

FAME analysis) and towards predominance of *Proteobacteria* rather than *Bacteroidetes* in the integrated livestock-cotton system appear to be associated to the higher enzyme activities, nutrient turnover and soil organic matter content found in this study compared to continuous cotton.

The correlations found between bacterial FAME indicators for G+, G– and actinomycetes and the predominant phyla found in this soil were interesting. For example, we found a positive correlation between *Actinobacteria* and FAME indicators for actinomycetes, suggesting that both methods, FAME and pyrosequencing, could provide an overall fair estimation of this microbial group in soil as affected by management. Similarly, we found a positive correlation between *Bacteroidetes* and *Nitrospira* with FAME indicators for G– populations. However, we found a negative correlation between *Proteobacteria* and FAME indicators for G– populations, while most members in this bacterial phyla are G– bacteria. Possible reasons are that the FAME-MIDI protocol used can extract FAMES not related to active microorganisms because of being attached (stored) to soil organic matter. Schutter and Dick (2000) reported that some FAME indicators for G– bacteria (i.e., i17:0 3OH) were extracted in significantly greater quantities ($P < 0.05$) with the MIDI protocol compared to an ester-linked (EL) method, which in theory can extract only ester-linked (not free) fatty acids from soil. However, this trend may be also influenced by the FAMES used for calculating the sum for G– bacteria. On the other hand, it was pointed out by Spain et al. (2009) that it is possible to obtain a larger proportion of *Proteobacteria* with pyrosequencing due to the use of small 16S rRNA gene fragments. The same group also pointed out that the majority of *Proteobacterial* clones (as well as others phyla) in soil data sets can belong to orders and families containing no described cultivated representatives (Elshahed et al., 2008; Spain et al., 2009).

5. Conclusions

Compared to a specialized system such as continuous cotton, pyrosequencing revealed significant changes in bacterial phyla distribution under an integrated livestock-cotton system for a semiarid soil after 10 years. Predominant bacteria were identified under the integrated livestock-cotton system (i.e., *Proteobacteria*) and in continuous cotton (i.e., *Bacteroidetes*) which may imply that these bacteria play major roles in soil processes under these different management systems. Different bacteria were predominant in non-grazed areas (i.e., *Proteobacteria* and *Gemmatimonadetes*) and grazed-areas (i.e., *Actinobacteria* and *Planctomycetacia*) of the integrated livestock-cotton system in response to possible differences in substrates amount and quality in soil, depending on the presence or absence of livestock activities. Bacteria with better adaptation to low amount or quality of substrates due to the lack of rhizosphere and/or extreme temperature changes were identified in this semiarid soil in terms of higher abundance (i.e., *Verrucomicrobiae*, *Acidobacteria* and *Thermomicrobia*) under fallow periods of the rotation than under cotton (Rye-Cotton-Wheat-Fallow or continuous cotton) and grass cover (pasture). This study also found positive correlations between certain bacterial phyla and other soil properties such as total C, microbial biomass C and enzyme activities of C and N cycling. The findings of this study provide some insights into key bacteria sustaining soil metabolic capacity or functioning which can open future avenues of research.

Acknowledgement

The authors would like to thank Mr. Jon Cotton for his technical assistance with soil sampling and DNA extraction.

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